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Modulation of DNA and protein adducts in smokers by genetic polymorphisms in *GSTM1*, *GSTT1*, *NAT1* and *NAT2*

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The formation of DNA and protein adducts by environmental pollutants is modulated by host polymorphisms in genes that encode metabolizing enzymes. In our study on 67 smokers, aromatic-DNA adduct levels were examined by nuclease P1 enriched ^{32}P-postlabelling in mononuclear blood cells (MNC) and 4-aminobiphenyl-haemoglobin adducts (4-ABP-Hb) by gas chromatography-mass spectroscopy. Genetic polymorphisms in glutathione *S*-transferase M1 (*GSTM1*), T1 (*GSTT1*) and *N*-acetyl-transferase 1 (*NAT1*) and 2 (*NAT2*) were assessed by polymerase chain reaction-based methods. DNA adduct levels, adjusted for the amount of cigarettes smoked per day, were higher in *GSTM1*(−/−) individuals (1.30 ± 0.57 adducts per 10^8 nucleotides) than in *GSTM1*(+) subjects (1.03 ± 0.56, $P = 0.05$), higher in *NAT1* slow acetylators (1.58 ± 0.54) than in *NAT1* fast acetylators (1.11 ± 0.58, $P = 0.05$) and were also found to be associated with the *NAT2* acetylator status (1.29 ± 0.64 and 1.03 ± 0.46, respectively, for slow and fast acetylators, $P = 0.06$). An effect of *GSTT1* was only found in combination with the *NAT2* genotype; individuals with the *GSTT1*(−/−) and *NAT2*-slow genotype contained higher adduct levels (1.80 ± 0.68) compared to *GSTT1*(+)/*NAT2* fast individuals (0.96 ± 0.36). Highest DNA adduct levels were observed in slow acetylators for both *NAT1* and *NAT2* also lacking the *GSTM1* gene (2.03 ± 0.17), and lowest in *GSTM1*(+) subjects with the fast acetylator genotype for both *NAT1* and *NAT2* (0.91 ± 0.45, $P = 0.01$). No overall effects of genotypes were observed on 4-ABP-Hb levels. However, in subjects smoking less than 25 cigarettes per day, 4-ABP-Hb levels were higher in *NAT2* slow acetylators (0.23 ± 0.10 ng/g Hb) compared to fast acetylators (0.15 ± 0.07, $P = 0.03$). These results provide further evidence for the combined effects of genetic polymorphisms in *GSTM1*, *GSTT1*, *NAT1* and *NAT2* on DNA and protein adduct formation in smoking individuals and indicate that, due to the complex carcinogen exposure, simultaneous assessment of multiple genotypes may identify individuals at higher cancer risk. Pharmacogenetics 11:389–398 © 2001 Lippincott Williams & Wilkins

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Introduction

Many cigarette smoke constituents are lipid soluble and tend to accumulate in organisms, unless they are metabolized to water soluble derivatives to facilitate excretion in urine or faeces. However, some derivatives are electrophilic and can bind to macromolecules such as DNA or proteins (Cooper & Grover, 1990). These carcinogen-DNA and carcinogen-protein adducts represent a complex continuum, including exposure, absorption, distribution, activation, detoxification and cell turnover (including DNA repair for DNA adducts). Therefore, the measurement of DNA and protein adducts has frequently been applied to assess human exposure to aromatic compounds (Schulte & Perera, 1993). However, adduct levels were found to vary significantly between individuals with apparently similar exposures and genetic polymorphisms in genes that code for metabolizing enzymes are thought to be involved (Bartsch & Hietanen, 1996).

When an electrophile forms a covalent bond with

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glutathione (GSH), it is no longer toxic and can be excreted. The reaction rate of GSH with electrophilic compounds can be enhanced by glutathione-*S*-transferases (GSTs) (Ketterer *et al.*, 1992). The m and q class GSTs (*GSTM1* and *GSTT1*) are polymorphic, containing expressing alleles [further indicated as *GSTM1*(+) or *GSTT1*(+), respectively] and null alleles [individuals lacking both alleles are indicated as *GSTM1*(−/−) or *GSTT1*(−/−), respectively] (Ketterer *et al.*, 1992). Detoxification of activated polycyclic aromatic hydrocarbons (PAH) is mediated by *GSTM1* (Ketterer *et al.*, 1992), whereas *GSTT1* seems to be involved in the detoxification of alkyl halides and lipid peroxides (Hayes *et al.*, 1995). It has been reported that subjects with the null genotypes of *GSTM1* and *GSTT1* may have an increased risk for developing, respectively, squamous cell carcinoma of the lung (D'Errico *et al.*, 1996) and colorectal cancers (Deakin *et al.*, 1996). Furthermore, subjects lacking the *GSTM1* locus seem to have higher PAH–DNA adduct levels in lung (Ketterer *et al.*, 1992; Shields *et al.*, 1993; Kato *et al.*, 1995; Rojas *et al.*, 1998). However, such a relationship could not consistently be established in peripheral blood cells (Grinberg-Funes *et al.*, 1994; Ichiba *et al.*, 1994; Hou *et al.*, 1995; Rothman *et al.*, 1995; Butkiewicz *et al.*, 1998; Rojas *et al.*, 1998). Little information is currently available regarding the impact of *GSTT1* on DNA adduct formation.

N-acetyltransferases (*NAT 1* and *2*), which are involved in the metabolism of aromatic amines (AA), are also genetically polymorphic and have been associated with increased risks for developing certain cancers in smokers. The most consistent findings for the *NAT2* polymorphism show that slow acetylators have an increased risk for developing bladder cancer, but have a lower risk for colorectal cancer (D'Errico *et al.*, 1996). In addition, *NAT1* seems to modulate the risks for some tobacco smoke related cancers; for example, long-time smokers who are homozygous mutant at the *NAT1* locus have a 27-fold increased risk of bladder cancer (Taylor *et al.*, 1995), whereas the *NAT1**10 allele (putatively fast acetylating) seems to be associated with a higher risk for colorectal cancer (Bell *et al.*, 1995). Generally, *N*-acetylation of AA is considered to be a detoxifying mechanism, since it competes with *N*-oxidation. On the other hand, acetylation might also be involved in the activation of xenobiotics, since hydroxylamines and hydroxamic acids can be further activated by both *NAT1* and *NAT2* via O-acetylation (Hein *et al.*, 1993). *NAT2* slow acetylators were found to have increased levels of haemoglobin adducts caused by 4-aminobiphenyl (4-ABP-Hb) (Vineis *et al.*, 1990; Shields, 1993; Vineis *et al.*, 1994; Yu *et al.*, 1994; Probst-Hensch *et al.*, 2000). However, others did not observe an effect of *NAT2* (Dallinga *et al.*, 1998) and recently also the *NAT1***10* allele did not seem to influence 4-ABP-Hb levels (Probst-Hensch *et al.*, 2000).

Overall, studies on genetic polymorphisms and their impact on DNA or protein adduct levels are not conclusive and, therefore, more studies are needed to further elucidate the role of genotypes in adduct formation. In the present study, we examined *GSTM1*, *GSTT1*, *NAT1* and *NAT2* genotypes in relation to aromatic DNA adduct levels in mononuclear blood cells (MNC) and haemoglobin adducts of 4-ABP in 67 smoking individuals, assessed by nuclease P1 enriched ^{32}P-postlabelling and gas chromatography-mass spectroscopy (GC-MS), respectively.

Materials and methods

Study population

Blood from 67 healthy smoking Caucasian volunteers (26 males, 41 females) with an average age of 41 ± 8 years was analysed. These individuals self-reported to smoke between 1 and 50 cigarettes per day (overall mean 25 ± 11 cigarettes per day) and reported not to be occupationally exposed to aromatic compounds. Informed consent was obtained from all individuals.

Isolation of mononuclear blood cells and erythrocytes

Ten ml of peripheral blood was sampled by venipuncture into EDTA-tubes. Granulocytes and erythrocytes were separated from mononuclear cells (monocytes plus lymphocytes) by centrifugation on lymphoprep [Lymphoprep™ (1.077 g/ml), Nycomed, Oslo, Norway] according to Bøyum (1976). The mononuclear cell-fraction was washed twice with PBS and stored at −20 °C until DNA isolation. The erythrocyte fraction was washed twice with 0.9% saline and frozen at −80 °C.

DNA isolation and ^{32}P-postlabelling

DNA was purified by a standard phenol extraction procedure as described previously (Godschalk *et al.*, 1998). The DNA was precipitated with two volumes of cold ethanol after addition of 1 : 30 volume 3 M sodium acetate, pH 5.3 and washed with 70% ethanol. Subsequently, DNA was dissolved in 2 mM Tris-buffer, pH 7.4. Concentration and purity were determined spectrophotometrically by absorbances at 230, 260 and 280 nm. The final volume was adjusted to achieve a DNA-concentration of approximately 2 mg/ml. Subsequently, the NP1-enriched

^{32}P-postlabelling assay was performed (Godschalk *et al.*, 1998) on all samples, at least in duplicate. Briefly, DNA (approximately 10 µg) was digested into nucleotide monophosphates and treated with nuclease P1 for 40 min at 37 °C. Then, nucleotides were 5′-labelled with ^{32}P using T4-polynucleotide kinase (5.0 U) for 30 min at 37 °C. Radiolabelled adduct nucleotide biphosphates were separated by chromatography on PEI-cellulose sheets (Machery Nagel, Düren, Germany). The following solvent systems were used: D1, 1 M NaH_2PO_4 pH 6.5; D2, 8.5 M urea, 5.3 M lithiumformate pH 3.5; D3, 1.2 M lithiumchloride, 0.5 M Tris, 8.5 M urea pH 8.0; D4, 1.7 M NaH_2PO_4 pH 6.0. In each experiment, three standards of [^{3}H]BPDE modified DNA with known modification levels (1 per 10^7, 10^8, 10^9 nucleotides) were run in parallel for quantification purposes. Quantification was performed by using phosphor imaging technology (Molecular Dynamics™, Sunnyvale, CA, USA). Detection limits of < 0.5 adducts per 10^8 nucleotides can be obtained for diagonal radioactive zones (DRZ).

Analysis of 4-ABP-Hb levels

4-ABP-Hb levels were determined by GC-MS as described by Bryant *et al.* (1987). Erythrocytes were lysed with three volumes ice-cold deionized water, centrifuged, and the Hb-containing solution was dialysed against deionized water for 48 h at 4 °C. The Hb concentration of the dialysate was determined using Drabkin's method (Sigma kit 525a, Sigma, St Louis, MO, USA). 4-ABP was released from the Hb by addition of 10 N NaOH. The dialysate was twice extracted with hexane and subsequently 4-ABP was derived by the addition of triethylamine and pentafluoropropionic anhydride. After evaporation of the hexane, the residue was redissolved in 20 µl dichloromethane, and analysed by GC-MS as described (Dallinga *et al.*, 1998).

Determination of genetic polymorphisms in GSTM1, GSTT1, NAT1 *and* NAT2

The *GSTM1* and *GSTT1* genotypes were determined using a polymerase chain reaction (PCR)-based assay as described by Oude Ophuis *et al.* (1998). PCR was carried out in a total volume of 25 µl, containing 0.1 µg DNA, 0.2 mM of each dNTP, 5 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 1 U of Taq-polymerase (Pharmacia Biotech, Roosendaal, The Netherlands), 0.2 µM of the *CYP1A1* (control) primers and 0.4 µM *GSTM1* or *GSTT1* primers. Exact information about the primers used for *GSTM1* can be found in Brockmöller *et al.* (1992) and for *GSTT1* in Pemble *et al.* (1994). After denaturation at 94 °C for 4 min, the PCR was followed by 35 cycles of amplification. The PCR product was analysed on a 2% agarose gel for the presence of *CYP1A1* bands at 204 bp (control) and 480 bp (*GSTT1*) or 650 bp (*GSTM1*).

In addition to the wild-type (wt) allele *NAT1*4*, seven variant *NAT1* alleles (*NAT1*3*, **10*, **11*, **14*, **15*, **17* and **22*) were analysed. *NAT1* alleles **15*, **17* and **22* were identified by conventional PCR-restriction fragment length polymorphism (RFLP) based methods, whereas, the polymorphic sites at nt 1088 and 1095 specific for *NAT1*10*, **14* and **11* were identified using LightCycler technology as previously described (Wikman *et al.*, 2001). In the PCR-RFLP method, the entire gene was first amplified with *NAT1* specific primers after which the 'long' PCR product was used as a template in consequent nested-PCRs using specific primers for each allele. After amplification, PCR products were cut with restriction enzymes and the bands produced were separated on agarose gels. In the LightCycler analysis, PCR and melting curve analyses were performed in 10 µl volumes in glass capillaries using: 1 × PCR buffer, 2.5–3 mM $MgCl_2$ 200 µM dNTPs, 0.1% BSA, 0.5 U Taq polymerase and 0.5 µM of each primer. An initial denaturation at 95 °C for 2 min was followed by 45 cycles of amplification. Melting curve analysis was performed by plotting the negative derivatives of fluorescence against temperature (−dF/dT). The alleles *NAT1*14*, **15*, **17* and **22* were considered to be phenotypically slow acetylating, whereas **10* was classified as a fast acetylating allele. *NAT1*4*, **3* and **11* were classified as normal acetylators. Classification of the subjects into fast (wt/fast or fast/fast), intermediate (wt/wt) or slow acetylators (slow/wt; no homozygous slow acetylators were found in this study population) was derived from the genotype data according to current knowledge on the functional activity of the variant alleles.

NAT2 genotyping was performed using standard PCR procedures (Doll *et al.*, 1995). PCR was carried out in a volume of 100 µl, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1.0 µg DNA, 0.2 mM of each dNTP, 1 µg of each primer and 2.5 U of Taq-polymerase. After 5 min, denaturation at 94 °C, DNA amplification was performed in 30 cycles. Digestion of 2 µl of the PCR product was carried out in a volume of 40 µl, using the appropriate buffers and the restriction enzymes *Msp*I and *Kpn*I, *Bam*HI and *Taq*I or *Dde*I. Digested samples were loaded on a 5% acrylamide gel containing ethidium bromide, run for 16 h at 30 V and visualized and photographed under ultraviolet light. Individuals were considered phenotypically 'fast' acetylators if they were carriers of at least one *NAT2*4* (wt) allele (Lin *et al.*, 1993).

Statistical analysis

Results are presented as mean ± SD. Non-parametric tests [Mann–Whitney *U*-test (two groups) or Kruskal–Wallis test (more than two groups)] were applied to evaluate statistical significance. No significant differences were observed in age and gender distribution between individuals with different genotype combinations and therefore adduct levels were only adjusted for the amount of cigarettes smoked per day. $P < 0.05$ was considered statistically significant.

Results

Distribution of genetic polymorphisms in GSTM1, GSTT1, NAT1 *and* NAT2

The *GSTM1* and *NAT2* genotypes could be assessed in the complete study population of 67 volunteers, whereas the *GSTT1* and *NAT1* genotypes could be assessed in, respectively, 58 and 56 individuals only (DNA no longer available for genotyping or absence of a PCR-product). The *GSTM1*(−/−) and *GSTM1*(+) genotypes were found in 35 and 32 individuals, respectively (52% and 48%). The *GSTT1*(−/−) and *GSTT1*(+) genotype were observed in 28% (16/58) and 72% (42/58), respectively. The *NAT1* 'slow', 'intermediate' and 'fast' acetylator genotypes were found in 11% (6/56), 62% (35/56) and 27% (15/56), respectively. The distribution of the *NAT2* genotype was 54% (36/67) and 46% (31/67) for slow and fast acetylators, respectively (Table 1). Five of the *NAT2* fast acetylators (7% of total population) were homozygous for *NAT2**4.

Overall analysis of DNA and protein adduct levels

DNA adduct analysis in MNC by thin layer chromatography resulted in DNA adduct profiles which are typical for exposure to cigarette smoke: so-called diagonal radioactive zones (DRZ) were observed in most cases and DNA adduct levels were determined by quantification of the total radioactivity in this DRZ. The mean total DNA adduct level in MNC was 1.22 ± 0.58 adducts per 10^8 nucleotides and ranged from < detection limit (adduct level set on 0.25 per 10^8 nucleotides) to 2.6 adducts per 10^8 nucleotides. A positive correlation was observed between the self-reported amount of cigarettes smoked per day and aromatic-DNA adduct levels in MNC ($r = 0.26$, $P = 0.04$). The mean level of the protein adduct, 4-ABP-Hb, was 0.19 ± 0.09 ng/g and ranged from 0.05–0.52 ng/g. Also, in this case, a significant correlation was observed between daily cigarette consumption and 4-ABP-Hb levels ($r = 0.36$, $P = 0.05$). The relatively low correlation coefficients indicate that large inter-individual variations were present. An overall correlation was observed between DNA adduct and 4-ABP-Hb levels ($r = 0.44$, $P = 0.0003$).

Impact of GSTM1, GSTT1, NAT1 *and* NAT2 *on DNA adduct levels in MNC*

Mean aromatic-DNA adduct levels, adjusted for the amount of cigarettes consumed per day, were significantly higher in individuals with the *GSTM1*(−/−) genotype (1.30 ± 0.57 adducts per 10^8 nucleotides) than in individuals who were *GSTM1*(+) (1.03 ± 0.56 adducts per 10^8 nucleotides, $P = 0.05$, Table 1). No such effect was observed for *GSTT1* (1.28 ± 0.60 and 1.12 ± 0.46 in, respectively, *GSTT1*(−/−) and *GSTT1*(+) subjects, $P = 0.41$). DNA adduct levels in *NAT1* slow acetylators (1.58 ± 0.54, $n = 6$) were higher than in intermediate (1.07 ± 0.62, $n = 35$) and fast acetylators (1.18 ± 0.53, $n = 15$). Since no differences were observed between putatively intermediate and fast acetylators, these two groups were combined for further analysis of genotype combinations (overall adduct level: 1.11 ± 0.58, $P = 0.05$, Table 1). Also, for *NAT2*, DNA adduct levels in MNC of slow-acetylators (1.29 ± 0.64, $n = 36$) tended to be higher than those found in heterozygous fast acetylators (1.07 ± 0.48, $n = 26$) and were even lower for homozygous fast acetylators (*NAT2**4/*4, 0.86 ± 0.39, $n = 5$, $P = 0.18$). For statistical reasons, homozygous and heterozygous fast acetylators were pooled and further referred to as fast acetylators (1.03 ± 0.46, $n = 31$, $P = 0.06$, Table 1).

Combinations of two genotypes resulted in overall differences which were in agreement with those expected for the individual genotypes (Table 2). For example, the effect of the combined *GSTM1* and *NAT2* genotypes on DNA adduct levels [1.55-fold higher in slow acetylators/*GSTM1*(−/−) compared to fast acetylators/*GSTM1*(+)], matched the effect that could be expected from the individual influences of both *GSTM1* (1.26-fold) and *NAT2* (1.25-fold): ($1.26 \times 1.25 = 1.58$). On the other hand, when *GSTT1* was combined with *NAT2*, an effect on DNA adduct levels was observed which was stronger than the impact of the two genotypes separately [*GSTT1*(−/−)/*NAT2* slow: 1.80 ± 0.68 adducts per 10^8 nt; *GSTT1*(+)/*NAT2* fast: 0.96 ± 0.36, $P < 0.01$]. The observed effect was 1.88-fold, whereas a 1.43-fold difference (1.14×1.25) was expected. The combined effect of the *NAT1* and *GSTT1* genotypes was lower than expected, but this could be due to the low number of individuals carrying the *NAT1* slow/*GSTT1*(−/−) genotype ($n = 2$).

Combinations of more than two genotypes resulted

Table 1. The effect of *GSTM1*, *GSTT1*, *NAT1* and *NAT2* polymorphisms on aromatic-DNA adduct levels in MNC, and 4-ABP-Hb levels in erythrocytes from smoking individuals (DNA and protein adduct levels were adjusted for the amount of cigarettes smoked per day)

	GSTM1			*GSTT1*			*NAT1*			*NAT2*		
	GSTM1(−/−)	*GSTM1*(+)	P	*GSTT1*(−/−)	*GSTT1*(+)	P	*Slow*	*Fast*	P	*Slow*	*Fast*	P
n	35 (52%)	32 (48%)		16 (28%)	42 (72%)		6 (11%)	50 (89%)		36 (54%)	31 (46%)	
DNA adducts	1.30 ± 0.57[a]	1.03 ± 0.56	0.05	1.28 ± 0.60	1.12 ± 0.46	0.41	1.58 ± 0.54	1.11 ± 0.58	0.05	1.29 ± 0.64	1.03 ± 0.46	0.06
4-ABP-Hb	0.18 ± 0.09[b]	0.17 ± 0.10	0.28	0.20 ± 0.11	0.16 ± 0.08	0.21	0.18 ± 0.10	0.18 ± 0.09	0.96	0.18 ± 0.10	0.17 ± 0.09	0.82

[a]Mean ± SD aromatic-DNA adduct level in adducts per 10[8] nucleotides. [b]Mean ± SD 4-ABP-Hb level in ng/g Hb.

in many missing values. Therefore, groups were again pooled according to the putatively 'high' and 'low risk' genotypes as shown in Table 3, i.e. group 1: *NAT* (1 and/or 2) slow acetylating and null for *GST* (M1 and/or T1) versus group 3: *NAT* fast acetylating and positive for GSTs. All other possible genotype combinations were combined as a group in between. In this case, highest DNA adduct levels were observed in slow acetylators for both *NAT1* and *NAT2*, also lacking the *GSTM1* gene (2.03 ± 0.17, $n = 3$), and lowest in *GSTM1*(+) subjects with the fast acetylator genotype for both *NAT1* and *NAT2* (0.91 ± 0.45, $P = 0.01$, $n = 9$, Table 3). Although only three individuals were found with the 'high risk' genotype combination *NAT1* slow/*NAT2* slow/*GSTM1*(−/−), all three contained DNA adduct levels that were above the 90th percentile of the total study population. On the other hand, DNA adduct levels in most individuals (seven out of nine) with the *NAT1* fast/*NAT2* fast/*GSTM1*(+) genotype did not go beyond the 50th percentile (= median) and adduct levels in none of these individuals went beyond the 75th percentile. Unfortunately, in this study population, no individuals were found who carried the *GSTM1*(−/−), *GSTT1*(−/−) and slow acetylating genotypes for *NAT1* as well as *NAT2*, but seven individuals were found who were positive for *GSTM1* and *GSTT1* and fast acetylating for both *NAT1* and *NAT2*. These individuals, however, did not contain the lowest DNA adduct levels (1.13 ± 0.34), indicating that the impact of *GSTT1* on the results of NP1-enriched ^{32}P-postlabelling is negligible.

Impact of GSTM1, GSTT1, NAT1 *and* NAT2 *on 4-ABP-Hb levels*

No effect of the *GSTM1* genotype was observed on 4-ABP-Hb levels (0.18 ± 0.09 ng/g and 0.17 ± 0.10 ng/g, for *GSTM1*(−/−) and *GSTM1*(+) individuals, respectively, $P = 0.28$). *GSTT1* was also not involved in the modulation of 4-ABP-Hb levels (0.20 ± 0.11 and 0.16 ± 0.08 for *GSTT1*(−/−) and *GSTT1*(+), respectively, $P = 0.21$). Furthermore, no overall effect of *NAT1* nor of *NAT2* on 4-ABP-Hb levels was observed (Table 1). Nonetheless, 4-ABP-Hb levels in homozygous *NAT2* fast acetylators (*NAT2**4/*4, 0.11 ± 0.04 ng/g, $n = 5$) tended to be lower than those in *NAT2* slow acetylators (0.18 ± 0.10, $P = 0.09$), whereas, 4-ABP-Hb levels in *NAT2* heterozygous fast subjects (0.19 ± 0.09, $n = 26$) were not different from those in slow acetylators. Genotype combinations were not found to significantly modulate the formation of 4-ABP-Hb (Tables 2 and Table 3).

When the analysis was restricted to individuals who smoked fewer than 25 cigarettes per day

Table 2. The effect of binary combinations of *GSTM1*, *GSTT1*, *NAT1* and *NAT2* on aromatic-DNA adducts in MNC and 4-ABP-Hb levels in erythrocytes from smoking individuals (six possible combinations). Adduct levels were adjusted for the amount of cigarettes consumed per day

I	*GSTM1*		*II*	*GSTM1*		*III*	*GSTM1*	
	(−/−)	*(+)*		*(−/−)*	*(+)*		*(−/−)*	*(+)*
GSTT1(−/−)			*NAT1* slow			*NAT2* slow		
n	13	3	*n*	4	2	*n*	20	17
DNA adducts	1.31 + 0.65[a]	1.14 + 0.29	DNA adducts	1.88 + 0.34	0.99 + 0.25	DNA adducts	1.41 ± 0.61	1.14 ± 0.66
4-ABP-Hb	0.23 ± 0.10[b]	0.09 ± 0.10	4-ABP-Hb	0.22 ± 0.09	0.10 ± 0.02	4-ABP-Hb	0.18 ± 0.08	0.17 ± 0.11
GSTT1(+)			*NAT1* fast			*NAT2* fast		
n	18	24	*n*	27	22	*n*	15	15
DNA adducts	1.27 ± 0.48	1.00 ± 0.42	DNA adducts	1.27 ± 0.54	0.99 ± 0.57*	DNA adducts	1.14 ± 0.51	0.91 ± 0.40
4-ABP-Hb	0.16 ± 0.07	0.16 ± 0.10	4-ABP-Hb	0.18 + 0.09	0.19 + 0.10	4-ABP-Hb	0.18 + 0.12	0.15 + 0.09
IV	*GSTT1*		*V*	*GSTT1*		*VI*	*NAT1*	
	(−/−)	*(+)*		*(−/−)*	*(+)*		*Slow*	*Fast*
NAT1 slow			*NAT2* slow			*NAT2* slow		
n	2	4	*n*	5	28	*n*	3	27
DNA adducts	1.11 ± 0.43	1.81 ± 0.45	DNA adducts	1.80 ± 0.68	1.20 ± 0.49	DNA adducts	2.03±0.17	1.17±0.65
4-ABP-Hb	0.18 ± 0.14	0.18 ± 0.10	4-ABP-Hb	0.23 ± 0.09	0.16 ± 0.09	4-ABP-Hb	0.21 ± 0.10	0.17 ± 0.10
NAT1 fast			*NAT2* fast			*NAT2* fast		
n	11	30	*n*	11	14	*n*	3	22
DNA adducts	1.27 ± 0.70	1.05 ± 0.42	DNA adducts	1.05 ± 0.39	0.96 ± 0.36*	DNA adducts	1.13 ± 0.31	1.03 ± 0.51*
4-ABP-Hb	0.22 ± 0.11	0.17 ± 0.09	4-ABP-Hb	0.19 ± 0.12	0.17 ± 0.08	4-ABP-Hb	0.16 ± 0.11	0.19 ± 0.09

[a]Mean ± SD aromatic-DNA adduct level in adducts per 108 nucleotides. [b]Mean ± SD 4-ABP-Hb level in ng/g Hb. *Significantly different compared to value in upper left-hand corner [i.c. (−/−)/(−/−) or (−/−)/slow or slow/slow].

Table 3. Combined impact of combinations of three genotypes (out of *GSTM1*, *GSTT1*, *NAT1* or *NAT2*) on aromatic-DNA adduct levels and 4-ABP-Hb levels in smoking volunteers

	DNA adduct level Adducts per 10^8 nt	*4-ABP-Hb ng/g Hb*	*n*
GSTM1, NAT1, NAT2			
(−/−), slow, slow	2.03 ± 0.17	0.21 ± 0.10	3
At least 1 fast or (+)	1.15 ± 0.60*	0.17 ± 0.10	43
(+), fast, fast	0.91 ± 0.45*	0.21 ± 0.06	9
GSTM1, GSTT1, NAT1			
(−/−) (−/−), slow	1.42	0.28	1
At least 1 fast or (+)	1.26 ± 0.57	0.17 ± 0.09	28
(+) (+), fast	1.01 ± 0.46	0.18 ± 0.10	18
GSTM1, GSTT1, NAT2			
(−/−) (−/−), slow	1.91 ± 0.72	0.26 ± 0.06	4
At least 1 fast or (+)	1.14 ± 0.46	0.16 ± 0.09	43
(+) (+), fast	1.00 ± 0.37	0.18 ± 0.08	11
NAT1, NAT2, GSTT1			
slow, slow (−/−)	NF	NF	0
At least 1 fast or (+)	1.21 ± 0.56	0.18 ± 0.10	38
Fast, fast (+)	0.98 ± 0.39	0.20 ± 0.08	9

*Significantly different compared to [(−/−) (−/−), slow] or [(−/−), slow, slow]. NF, No individuals found with this particular genotype combination.

(median amount of cigarettes smoked per day), 4-ABP-Hb levels were significantly lower in *NAT2* fast acetylators (0.15 ± 0.07) than in slow acetylators (0.23 ± 0.10, $P = 0.03$). Surprisingly, in individuals who smoked 25 cigarettes per day or more, the opposite was found; 0.13 ± 0.06 for slow acetylators and 0.19 ± 0.11 for fast acetylators ($P = 0.12$), respectively. A similar effect was not observed for *NAT1*.

Discussion

Epidemiological studies indicate that the assessment of genetic polymorphisms in genes encoding for phase II enzymes may allow the identification of cancer susceptible individuals or subgroups (Bartsch & Hietanen, 1996; D'Errico *et al.*, 1996). It is thought that these polymorphisms affect cancer risk via their impact on DNA adduct formation, but the scientific literature is not consistent (Shields *et al.*, 1993; Rojas *et al.*, 1998; Schocket *et al.*, 1998). One possible reason for this inconsistency is that human exposure to carcinogens is often to complex chemical mixtures, which contain compounds that are differentially metabolized and/or detoxified. Therefore, it seems necessary to assess multiple polymorphisms to obtain a more clear view on the modulation of DNA or protein adduct formation by genetic polymorphisms. In the present study on smoking volunteers, we investigated the formation of aromatic-DNA adducts in MNC (determined by NP1-enriched ^{32}P-postlabelling) and 4-ABP-Hb adducts in erythrocytes (determined by GC-MS) and related adduct levels to genetic polymorphisms in *GSTM1*, *GSTT1*, *NAT1* and *NAT2*. The genetic polymorphism in the phase I enzyme *CYP1A1* (Ile/Val and MspI) was also assessed in the volunteers of this study, but *CYP1A1* mutant alleles were rare and did not interfere with the present results (data not shown).

DNA and protein adduct levels have frequently been used to assess the level of exposure to cigarette smoke constituents. We also observed significant relationships between the amount of cigarettes smoked per day and aromatic-DNA adduct levels in MNC and 4-ABP-Hb levels in erythrocytes. However, the correlation coefficients were low, which indicates that cigarette-dose alone is not sufficient to explain the observed inter-individual variations in adduct levels and thus genetic polymorphisms may be involved. Aromatic-DNA adduct levels in MNC of smoking *GSTM1*(−/−) subjects were modestly, but significantly, higher than in *GSTM1*(+) individuals. This is in agreement with previous studies (Rojas *et al.*, 1998; Butkiewicz *et al.*, 1998), whereas others

did not observe such an association (Grinberg-Funes *et al.*, 1994; Rothman *et al.*, 1995). It should be mentioned that the nuclease P1 enriched ^{32}P-postlabelling assay is a relatively non-specific assay, which detects many structurally unknown DNA adducts. Therefore, in the present study, the effects of genetic polymorphisms on adduct formation by specific compounds may be underestimated. An assay for the specific detection of benzo(a)pyrene-diol epoxide-DNA adducts was used in a recent study, and the effect of *GSTM1* on DNA adduct formation in MNC and human lung was indeed much clearer (Rojas *et al.*, 1998).

We also observed effects of *NAT1* and *NAT2* on DNA adduct levels in MNC. This is an indication for the presence of AA-derived DNA adducts in MNC of smokers, because especially AA are detoxified by *N*-acetylation. Our previous results already suggested that the formation of DNA adducts by AA in MNC of smoking individuals can be substantial, because six-fold higher adduct levels were observed by butanol enriched ^{32}P-postlabelling compared to NP1 enriched ^{32}P-postlabelling (Godschalk *et al.*, 1998). Generally, DNA adducts formed by AA are thought to be sensitive towards the action of nuclease P1, and are lost during the enrichment with NP1, which was used in the present study. However, Gupta *et al.* (1988) showed that AA bound to the exocyclic position of guanine and adenine are relatively resistant towards the action of NP1. A relationship between aromatic-DNA adducts and *NAT2* was also reported by Peluso *et al.* (1998) who reported higher adduct levels determined by NP1-enriched ^{32}P-postlabelling in slow acetylators than in fast acetylators. In the present study, DNA as well as protein adduct levels, were lower in homozygous *NAT2* fast acetylators (*NAT2*4/*4*) than in heterozygous fast acetylators (only one **4* allele), suggesting that this discrimination is recommended for future research. While Bell *et al.* (1995) identified the *NAT1*10* allele as fast acetylating, more recently, this finding could not be reproduced in a study investigating *NAT1* genotype–phenotype correlations (Bruhn *et al.*, 1999). However, because there was no difference in adduct levels between putatively *NAT1* fast and intermediate acetylators, and these two groups were actually pooled for the analysis, this dispute does not influence the conclusions drawn from our results.

In a study by Hou *et al.* (1995), an effect of *NAT2* on DNA adduct levels in bus maintenance workers was observed only in combination with the *GSTM1* genotype. Also, in this study, we observed a joint effect of *NAT2* and *GSTM1*, showing that adduct levels were higher in *GSTM1*(−/−)/*NAT2* slow individuals than in *GSTM1*(+)/*NAT2* fast subjects, but there was no evidence for a synergistic effect. On the other hand, the combination of *GSTT1*(−/−) and *NAT2* slow acetylators resulted in adduct levels which were higher than those expected from the effect of the two genes separately. This seems to be in line with a study by Welfare *et al.* (1999), who reported that *NAT2* or *GSTT1* had no effect on colorectal cancer, but their combination resulted in a significantly increased risk. Because little is known about the impact of *GSTT1* on adduct formation as determined by ^{32}P-postlabelling, more studies are necessary to elucidate the role of *GSTT1* in adduct formation and to confirm its interaction with *NAT2*.

Highest DNA adduct levels were observed in those individuals who carried the *GSTM1*(−/−), *NAT1* slow and *NAT2* slow genotypes (Table 3), which indicates that the assessment of multiple genotypes is actually necessary to identify those individuals at higher risk for the development of smoking related malignancies. Although the use of multiple genotypes in epidemiological studies will probably result in much higher risk estimates, such a study will lose statistical power, ending up with statistically non-significant results. Studies on DNA adduct formation, similar to the present study, might be helpful, since increased DNA adduct levels have recently been shown to implicate a higher cancer risk (Tang *et al.*, 2000; Vineis & Perera, 2000). Thus, molecular epidemiological studies assessing multiple genotypes currently have disadvantages, but this will need to change in the future, because our results reemphasize that such studies are actually necessary to improve the identification of individuals at a higher cancer risk.

Previous studies suggested that the *NAT2* polymorphism may affect 4-ABP-Hb levels and 4-ABP-DNA levels in bladder cells (Vineis *et al.*, 1990, 1994), but the results are inconsistent (Dallinga *et al.*, 1998; Martone *et al.*, 1998). In addition, the *GSTM1* genotype has been associated with the detoxification of 4-ABP (Yu *et al.*, 1995) but, again, the results have not been reproduced (Dallinga *et al.*, 1998). In the present study, no clear modulation of 4-ABP-Hb levels was observed by *GSTM1*, *GSTT1*, *NAT1* nor by *NAT2* or any combination of these polymorphisms. However, when the analysis was restricted to volunteers who smoked fewer than 25 cigarettes per day, adduct levels were lower in *NAT2* fast acetylators than in slow acetylators. Indeed, the *NAT2* polymorphism seems to be more relevant at low exposures compared to high levels of exposure (Vineis *et al.*, 1994). In individuals who smoked more than 25 cigarettes per day, 4-ABP-Hb levels tended to be modulated by *NAT2* in exactly the opposite direction. The role of *NAT1* and *NAT2* in the

^{32}P-postlabelling assay was performed (Godschalk *et al.*, 1998) on all samples, at least in duplicate. Briefly, DNA (approximately 10 µg) was digested into nucleotide monophosphates and treated with nuclease P1 for 40 min at 37 °C. Then, nucleotides were 5'-labelled with ^{32}P using T4-polynucleotide kinase (5.0 U) for 30 min at 37 °C. Radiolabelled adduct nucleotide biphosphates were separated by chromatography on PEI-cellulose sheets (Machery Nagel, Düren, Germany). The following solvent systems were used: D1, 1 M NaH_2PO_4 pH 6.5; D2, 8.5 M urea, 5.3 M lithiumformate pH 3.5; D3, 1.2 M lithiumchloride, 0.5 M Tris, 8.5 M urea pH 8.0; D4, 1.7 M NaH_2PO_4 pH 6.0. In each experiment, three standards of [^{3}H]BPDE modified DNA with known modification levels (1 per 10^7, 10^8, 10^9 nucleotides) were run in parallel for quantification purposes. Quantification was performed by using phosphor imaging technology (Molecular Dynamics™, Sunnyvale, CA, USA). Detection limits of < 0.5 adducts per 10^8 nucleotides can be obtained for diagonal radioactive zones (DRZ).

Analysis of 4-ABP-Hb levels

4-ABP-Hb levels were determined by GC-MS as described by Bryant *et al.* (1987). Erythrocytes were lysed with three volumes ice-cold deionized water, centrifuged, and the Hb-containing solution was dialysed against deionized water for 48 h at 4 °C. The Hb concentration of the dialysate was determined using Drabkin's method (Sigma kit 525a, Sigma, St Louis, MO, USA). 4-ABP was released from the Hb by addition of 10 N NaOH. The dialysate was twice extracted with hexane and subsequently 4-ABP was derived by the addition of triethylamine and pentafluoropropionic anhydride. After evaporation of the hexane, the residue was redissolved in 20 µl dichloromethane, and analysed by GC-MS as described (Dallinga *et al.*, 1998).

Determination of genetic polymorphisms in GSTM1, GSTT1, NAT1 *and* NAT2

The *GSTM1* and *GSTT1* genotypes were determined using a polymerase chain reaction (PCR)-based assay as described by Oude Ophuis *et al.* (1998). PCR was carried out in a total volume of 25 µl, containing 0.1 µg DNA, 0.2 mM of each dNTP, 5 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 1 U of Taq-polymerase (Pharmacia Biotech, Roosendaal, The Netherlands), 0.2 µM of the *CYP1A1* (control) primers and 0.4 µM *GSTM1* or *GSTT1* primers. Exact information about the primers used for *GSTM1* can be found in Brockmöller *et al.* (1992) and for *GSTT1* in Pemble *et al.* (1994). After denaturation at 94 °C for 4 min, the PCR was followed by 35 cycles of amplification. The PCR product was analysed on a 2% agarose gel for the presence of *CYP1A1* bands at 204 bp (control) and 480 bp (*GSTT1*) or 650 bp (*GSTM1*).

In addition to the wild-type (wt) allele *NAT1*4*, seven variant *NAT1* alleles (*NAT1*3*, **10*, **11*, **14*, **15*, **17* and **22*) were analysed. *NAT1* alleles **15*, **17* and **22* were identified by conventional PCR-restriction fragment length polymorphism (RFLP) based methods, whereas, the polymorphic sites at nt 1088 and 1095 specific for *NAT1*10*, **14* and **11* were identified using LightCycler technology as previously described (Wikman *et al.*, 2001). In the PCR-RFLP method, the entire gene was first amplified with *NAT1* specific primers after which the 'long' PCR product was used as a template in consequent nested-PCRs using specific primers for each allele. After amplification, PCR products were cut with restriction enzymes and the bands produced were separated on agarose gels. In the LightCycler analysis, PCR and melting curve analyses were performed in 10 µl volumes in glass capillaries using: 1 × PCR buffer, 2.5–3 mM $MgCl_2$ 200 µM dNTPs, 0.1% BSA, 0.5 U Taq polymerase and 0.5 µM of each primer. An initial denaturation at 95 °C for 2 min was followed by 45 cycles of amplification. Melting curve analysis was performed by plotting the negative derivatives of fluorescence against temperature (−dF/dT). The alleles *NAT1*14*, **15*, **17* and **22* were considered to be phenotypically slow acetylating, whereas **10* was classified as a fast acetylating allele. *NAT1*4*, **3* and **11* were classified as normal acetylators. Classification of the subjects into fast (wt/fast or fast/fast), intermediate (wt/wt) or slow acetylators (slow/wt; no homozygous slow acetylators were found in this study population) was derived from the genotype data according to current knowledge on the functional activity of the variant alleles.

NAT2 genotyping was performed using standard PCR procedures (Doll *et al.*, 1995). PCR was carried out in a volume of 100 µl, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1.0 µg DNA, 0.2 mM of each dNTP, 1 µg of each primer and 2.5 U of Taq-polymerase. After 5 min, denaturation at 94 °C, DNA amplification was performed in 30 cycles. Digestion of 2 µl of the PCR product was carried out in a volume of 40 µl, using the appropriate buffers and the restriction enzymes *Msp*I and *Kpn*I, *Bam*HI and *Taq*I or *Dde*I. Digested samples were loaded on a 5% acrylamide gel containing ethidium bromide, run for 16 h at 30 V and visualized and photographed under ultraviolet light. Individuals were considered phenotypically 'fast' acetylators if they were carriers of at least one *NAT2*4* (wt) allele (Lin *et al.*, 1993).

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